Diabetes primes neutrophils to undergo NETosis, which impairs wound healing

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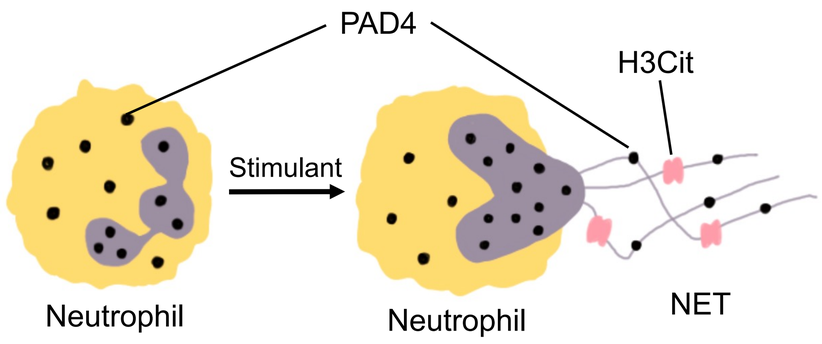
Background

- Diabetes compromises wound healing.
- One contributory mechanism is the formation of neutrophils extracellular traps (NETs).
- NETs consists of extracellular DNA, histones, and proteases.
- Formation of NETs (NETosis) requires histone citrullination by the enzyme peptidyl arginine deiminase 4 (PAD4) encoded by Padi4.
- PAD4 activity and levels of citrullinated histone (H3Cit) can be used to measure NETosis.



Methods

Human blood cell samples and NETosis stimulation
Blood samples from ten healthy individuals and ten diabetic subjects were obtained. Calcium ionophore ionomycin was used to stimulate NETosis in isolated neutrophils from the blood samples.

Induction of diabetic mouse model and NETosis stimulation
Streptozotocin (STZ) which is toxic to insulin-producing beta-cells in the pancreas was injected to induce type 1 diabetes in mice models. Immunofluorescence wide-field and confocal microscopy was used to examine the fresh blood cells from STZ-induced diabetic mice for H3Cit and NETs. Lipopolysaccharides (LPS), an exogenous pyrogen, was used to stimulate NETosis.

Wounding and histological examination
PAD4 gene was knocked out of mice. Wounds were then made on mice with full-thickness excisions; Histology was taken via controlled punch biopsies which were then stained with hematoxylin and eosin (H&E) and viewed under the microscope.

Immunofluorescence and confocal microscopy for macroscopic healing assessment
Immunofluorescence was done by incubating histological specimens with primary antibodies against H3Cit. To assess gross healing of wounds, photographs were also taken and compared to wound area on day 0 as a percentage.

DNase 1 treatment
Normoglycemic and diabetic WT mice were injected with DNase 1 whereas control mice were injected with vehicle. Wounds were then stained with H&E and observed under a microscope for re-epithelization analysis.

Results and Discussion

Neutrophils of diabetics are more susceptible to NETosis in vitro.
When stimulated, neutrophils from diabetics were more susceptible to NETosis compared to neutrophils from healthy controls (Fig 1).

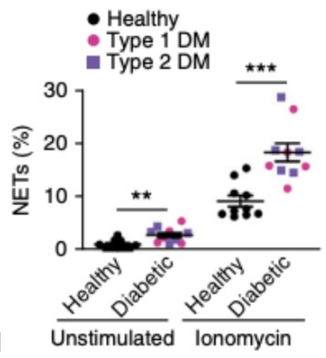


Fig. 1

Replicating results in mice models
STZ-induced mice were used to represent mice with diabetes type 1. When NETosis is induced with LPS, immunofluorescence showed an increase in amounts of H3Cit and NETs formed in STZ-induced mice compared to vehicle controls (Fig 2).

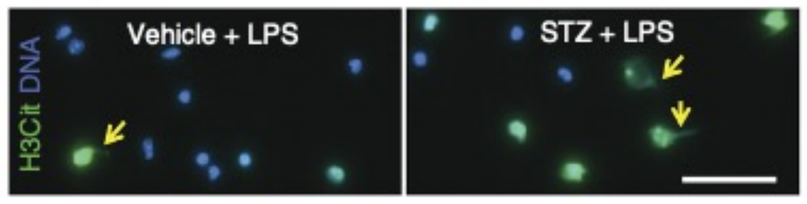


Fig. 2

PAD4-deficient mice improved wound healing
H&E staining and confocal microscopy showed decreased extracellular DNA in Padi4 knockout mice. Confocal microscopy also showed a lack of H3Cit in wounds of Padi4-/- mice (Fig 3). Hence, confirming the lack of NETosis without PAD4. Padi4-/- mice showed faster healing of wounds over 7 days (Fig 4).

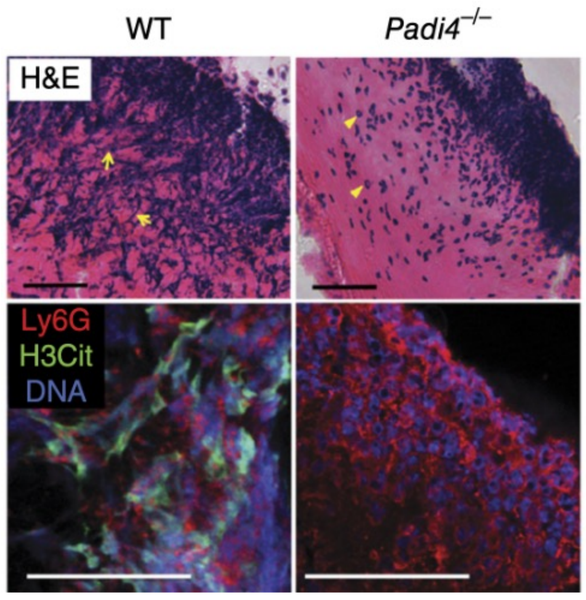


Fig. 3

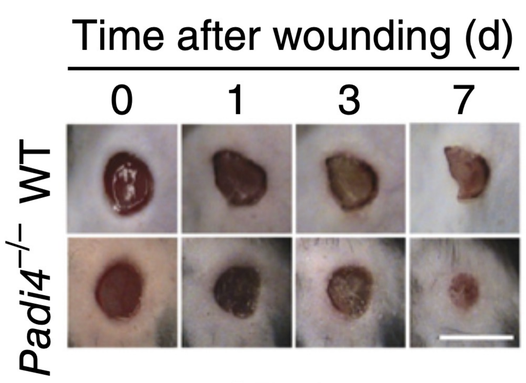


Fig. 4

Comparing wound healing of STZ-induced diabetic WT mice and PAD4-/- mice.
Diabetic WT mice had prolonged wound healing (Fig 5a) whereas NETosis delayed wound healing in diabetic mice (Fig 5b). When NETs was absent, diabetic and normoglycemic mice had no change in wound healing (Fig 5c).

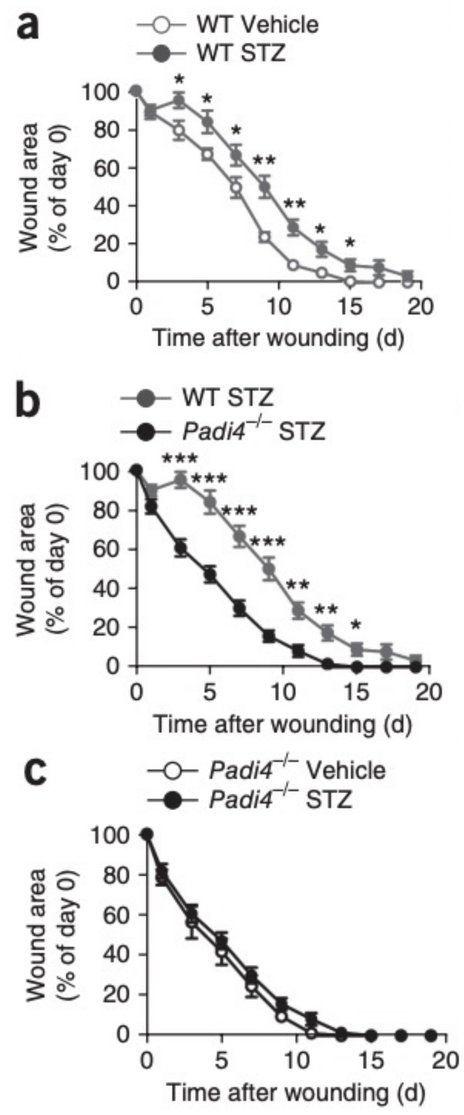


Fig 5

Effect of DNase 1 on wound healing in mice
DNase 1 facilitates the breakdown and removal of NETs, enhancing wound healing. WT diabetic mice treated with DNase 1 had a smaller wound area and more re-epithelisation as compared to those not treated. In contrast, DNase 1 treatment did not further improve wound healing in diabetic Padi4-/- mice (Fig 6). DNase 1 facilitates wound healing via the breakdown and removal of NETs.

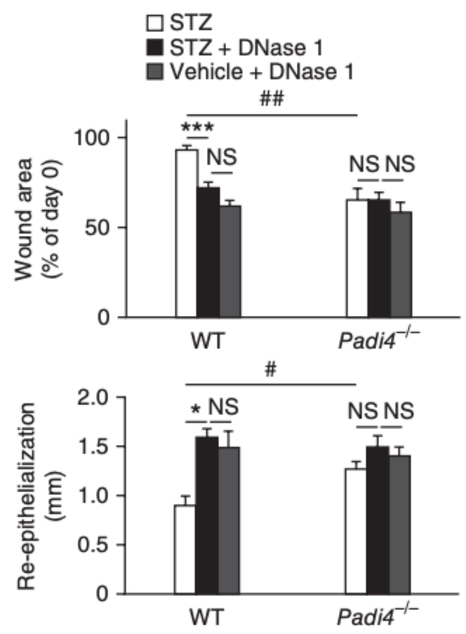


Fig 6

Conclusion

- Diabetes causes increased NETosis of neutrophils, of which PAD4 and H3Cit+ can be used as markers.
- Mice models which also shows increase in H3Cit+ in hyperglycaemia can be used.
- NETosis is a key component in delayed wound healing.

Future Direction

- DNase 1 could be further developed into a treatment for diabetic wounds.
- PAD4 inhibition has great potential to be a novel therapeutic strategy for improving wound healing as it targets the root problem of NETosis
- Anti-NET therapy is potentially beneficial in other diseases including inflammatory and thrombotic diseases in diabetic individuals.